

## A Constitutive Damage-Specific DNA-Binding Protein Is Synthesized at Higher Levels in UV-Irradiated Primate Cells

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Using a DNA band shift assay, we have identified a DNA-binding protein complex in primate cells which is present constitutively and has a high affinity for UV-irradiated, double-stranded DNA. Cells pretreated with UV light, mitomycin C, or aphidicolin have higher levels of this damage-specific DNA-binding protein complex, suggesting that the signal for induction can either be damage to the DNA or interference with cellular DNA replication. Physicochemical modifications of the DNA and competition analysis with defined substrates suggest that the most probable target site for the damage-specific DNA-binding protein complex is a 6-4'-(pyrimidine-2'-one)-pyrimidine dimer: specific binding could not be detected with probes which contain -TT- cyclobutane dimers, and damage-specific DNA binding did not decrease after photoreactivation of UV-irradiated DNA. This damage-specific DNA-binding protein complex is the first such inducible protein complex identified in primate cells. Cells from patients with the sun-sensitive cancer-prone disease, xeroderma pigmentosum (group E), are lacking both the constitutive and the induced damage-specific DNA-binding activities. These findings suggest a possible role for this DNA-binding protein complex in lesion recognition and DNA repair of UV-light-induced photoproducts.

DNA-binding proteins are cellular components involved in gene regulation, chromatin structure, nucleic acid synthesis and degradation, and nucleotide modification (32). They can recognize a primary sequence, a particular structure, or a specific conformation of DNA. Since DNA is frequently subject to damage by environmental and endogenous factors, the recognition and repair of DNA lesions by cellular components are integral parts of conservation of the genome.

In bacteria, the DNA repair pathways include a number of DNA damage recognition proteins and enzymes which work in concert to recognize, remove, and repair a variety of DNA lesions. Many of these repair functions are induced *de novo* in damaged cells as a part of the SOS pathway which is believed to facilitate the recovery of bacterial cells from the toxic effects of DNA damage (36, 37). Specific knowledge of the DNA lesion recognition and DNA repair pathways in mammalian cells, on the other hand, is limited. Complementation analysis of cells from patients with DNA repair disorders, such as xeroderma pigmentosum (XP), has indicated that the repair of bulky DNA lesions in mammalian cells is considerably more complex than that in bacteria. Several damage-specific DNA-binding (DDB) proteins in mammalian cells and tissues have been described (8-10, 25, 34), but the physiological roles of these proteins have not been determined. Since isolation and cloning of the genes which encode mammalian DNA repair proteins have met with little success, specific information about the formation of DNA repair complexes (in particular those involved in the repair of bulky lesions, such as UV photoproducts) and their regulation in a stress-induced environment is not yet available. Recent studies have indicated, however, that SOS-like inducible phenomena associated with DNA processing occur in mammalian cells exposed to carcinogens or tumor promoters (for a review, see references 5 and 7 and references therein; 26, 29, 35). Some of the proteins induced in these

cells, including DNA ligase (22) and O<sup>6</sup>-methylguanine-DNA-methyltransferase (19), are involved in DNA repair. Whether these proteins participate in a true mammalian SOS response remains to be determined, as does the biological role of these inducible responses.

Recently, we detected an enhanced repair of UV-damaged expression vectors in monkey cells pretreated with UV light or mitomycin C (26). We hypothesized that some of the cellular proteins which are associated with the processing of UV-damaged DNA might be enhanced in pretreated cells. We have used a DNA band shift assay and cell extracts to look for the presence and levels of UV-lesion-specific DNA-binding proteins in control and pretreated cells. Here we describe the identification of a constitutive DDB protein complex in control cells, its substrate specificity, and its enhanced synthesis by agents which either damage DNA or inhibit DNA replication. Since this DDB protein complex appears to bind specifically to 6-4'-(pyrimidine-2'-one)-pyrimidine dimers [(6-4) dimers] in UV-damaged DNA and cannot be detected in XP group E cells [which are deficient in (6-4) dimer removal (23)], we believe that it may have a role in UV lesion recognition and repair.

### MATERIALS AND METHODS

**Cells and cell treatments.** The African green monkey kidney cell line CV-1 was obtained from B. Howard (National Cancer Institute, Bethesda, Md.). The human cell lines, normal fetal fibroblast GM0011, normal skin fibroblasts GM01652A and GM00037D, and XP skin fibroblast GM02415 were purchased from the Human Genetic Mutant Cell Repository (Camden, N.J.). Cells were grown at 10% CO<sub>2</sub> in Dulbecco modified Eagle medium supplemented with 20 mM L-glutamine-10% fetal calf serum and without antibiotics.

Cells were treated with UV light or mitomycin C as described previously (26). The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (20 ng/ml) (33), aphidicolin (5 μM), dactinomycin (2.5 or 5 μg/ml), or cycloheximide (25

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Sequence	Name
5'-GATCTGATTCCCATCTCCTCAGTTTCACTTCTGCACCGCATG-3' 3'-ACTAAGGGGTAGAGGAGTCAAAGTGAAGACGTGGC-5'	3/4
-----GAC----- -----CTG-----	23/24
-----TC----- -----CT-----	3/134
-----GAC----- -----AAA-----	23/4
5'-GCATGCGCATATACGCACGTATATGCGCGCATACGCGCATATGC-3' 3'-CGTACGCGTATATGCGTGCATATACGCGCGTATGCGCGTATACG-5'	190/200
5'-GCAAGT <sup>^</sup> TGGAGGCAAGT <sup>^</sup> TGGAGGCAAGT <sup>^</sup> TGGAGGCAAGT <sup>^</sup> TGGAG-3' 3'-CGTTCA-ACCTCCGTTC-ACCTCCGTTC-ACCTCCGTTC-ACCTC-5'	11 <sup>^</sup> /44
5'-GCGCATGCATATACGCACGCAAGT <sup>^</sup> TGGAGCATACGCGCATATGC-3' 3'-CGGTACGTATATGCGTGGCTTC-ACCTCGTATGCGCGTATACG-5'	11 <sup>^</sup> /230

FIG. 1. Oligonucleotides used in DNA-binding studies.

µg/ml) (11, 14) was added to the growth medium, and cells were incubated for 12 to 24 h. Cells were then washed twice with phosphate-buffered saline, and fresh medium was added. Cells treated with 10 to 12 J of UV light per m<sup>2</sup>, 1 µg of mitomycin C per ml, or 5 µM aphidicolin appeared morphologically similar 24 h after treatment: flat, enlarged in size, and growth arrested. By 72 h, most of the cells resumed growth. These treatments usually produced little or no cell detachment from the dishes. Cells treated with TPA appeared rounded for the first 24 h and then reverted to the normal phenotype. Cells (10 to 20%) were detached by the end of 48 h; the rest recovered and continued growth. Dactinomycin arrested cells within 12 h after treatment, and the cells did not resume growth by the time of harvesting. To arrest growth, cells were washed twice with phosphate-buffered saline (mock treatment) and fed growth medium supplemented with 0.5% fetal calf serum. By 48 h, 80% of the cells were growth arrested, with no cell detachment.

**DNA.** Oligonucleotides used either as probes or competitors are shown in Fig. 1. Oligonucleotide 3/4 was chosen initially because it is rich in adjacent pyrimidines which are substrates for UV-induced pyrimidine dimers. Oligonucleotides were synthesized with a Vega automated synthesizer Coder 300 or a Biosearch model 8700 multiple-column DNA synthesizer and were kindly provided by E. Appella, J. Jones, and L. Neuhold (National Institutes of Health, Bethesda, Md.). The 11 mers without or with a single cyclobutane -TT- pyrimidine dimer (5'-GCAAGT<sup>^</sup>TGGAG-3) (2) were a generous gift from C. W. Lawrence (University of Rochester School of Medicine and Dentistry, Rochester, N.Y.).

Oligonucleotides were 5' end labeled with polynucleotide kinase, using [<sup>32</sup>P]ATP (5,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), annealed with the complementary strand, and ligated with T4 DNA ligase when necessary. The purity of the probes was analyzed on 20% polyacrylamide gels. Double-stranded 11<sup>^</sup>/44 mers containing at least one and a maximum of four -TT- pyrimidine dimers were gel purified before being used in binding assays. Labeled probes had an average specific activity of 2,000 cpm/fmol of DNA.

The synthetic polydeoxynucleotides poly(dI-dC) · poly(dI-dC), poly(dG) · poly(dC), and poly(dA) · poly(dT) were purchased from Pharmacia, Inc., Piscataway, N.J.

**DNA-binding assay.** The DNA-binding assay and DNA band shift analysis were modifications of the methods of Fried and Crothers (12) and Garner and Revzin (13). Whole-cell extracts were prepared as described by Manley et al.

(21) as modified by Wood et al. (38). Nuclear extracts were prepared by the method of Lee et al. (18). For both procedures, cells were washed twice with ice-cold phosphate-buffered saline, gently scraped off the dishes, and collected by centrifugation. Phosphate-buffered saline was removed, and cells were suspended in cell lysis buffer and processed according to the published methods. The protein concentration in different cell extract preparations was between 5 and 10 mg/ml. Portions (3 to 6 µg each) of cell or nuclear extract were incubated with 10 to 15 fmol of labeled probe and 2 µg of poly(dI-dC) · poly(dI-dC) in a buffer containing 150 mM NaCl-20 mM Tris hydrochloride (pH 7.5)-0.2 mM EDTA-1 mM MgCl<sub>2</sub>-1 mM dithiothreitol-5% glycerol in a total volume of 20 µl. The mixture was incubated on ice for 20 to 30 min and then analyzed on 4% nondenaturing polyacrylamide gels.

Electrophoresis was performed in 40 mM Tris hydrochloride (pH 8.3)-40 mM boric acid-0.4 mM EDTA at 150 V for 1 h at room temperature. Gels were briefly fixed in 15% acetic acid-10% methanol, dried, and exposed to Kodak XAR-2 film. Scanning of X-ray films was performed on an LKB 2202 Ultrosan laser densitometer. Densitometry was performed within the linear exposure range of the X-ray films. Because of irregularities in band shape, the scans were performed through different regions of the bands and the results were averaged. Binding activity is expressed as peak area and represents an absolute value for a particular experiment, since the same amount of protein was used for each data point.

**Modification of DNA.** (i) **UV damage.** DNA was irradiated on ice with a 254-nm light source at 1 to 25 kJ/m<sup>2</sup> as described previously (27). UV-induced pyrimidine dimers were photoreactivated with *Escherichia coli* photolyase (kindly provided by A. Sancar, University of North Carolina School of Medicine) as reported previously (28). To prepare UV-irradiated DNA with the cytosine hydrates eliminated, oligonucleotides were incubated after irradiation for 60 min at 60°C (17).

(ii) **Oxidative damage.** Oxidative lesions were induced by incubating oligonucleotides either with 30 µM to 30 mM hydrogen peroxide and 0.1 mM cupric sulfate for 30 min at 37°C or with 0.5 to 10 mg of osmium tetroxide per ml for 5 to 20 min at 70°C (15).

(iii) **Mismatched DNA.** Double- and triple-mismatched oligonucleotides (Fig. 1, 3/134 and 23/4, respectively) were prepared by annealing oligonucleotides 3 and 4 with their complementary strands 134 and 23 containing double- or triple-base substitutions, respectively.

UV-irradiated or chemically treated oligonucleotides were analyzed for the extent of modifications on either nondenaturing 20% polyacrylamide gels or denaturing 20% polyacrylamide-7 M urea gels. Analysis of oligonucleotides for the presence of T4 polymerase-exonuclease blocking sites and hot alkali labile sites was carried out as described by Doetsch et al. (6).

## RESULTS

**Detection of DDB protein complex in extracts from monkey CV-1 cells.** We questioned whether mammalian cells contain a constitutive DNA-binding protein(s) specific for UV-irradiated DNA rather than a particular DNA sequence. We prepared CV-1 whole-cell extracts as described by Wood et al. (38), since such extracts apparently contain all cellular proteins needed to support UV-induced repair synthesis in vitro. We used these extracts with a UV-irradiated DNA

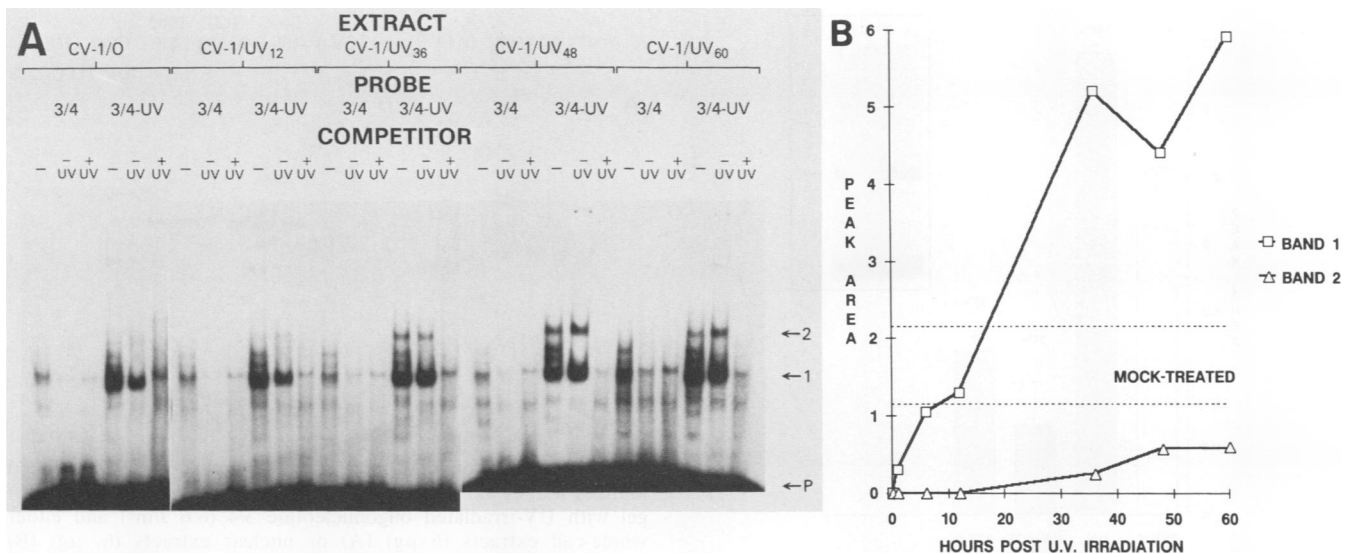


FIG. 2. Time course of induction of DDB protein complex in CV-1 cells pretreated with UV light. (A) Mobility shift gel with unirradiated (3/4) or UV-irradiated ( $8.6 \text{ J/m}^2$ ) (3/4-UV) oligonucleotide probe and  $6.6 \mu\text{g}$  of cell extract proteins in the absence (–) or presence (+) of excess competitor 3/4 DNA (unirradiated, –UV; UV-irradiated, +UV). Whole-cell extracts were prepared from mock-treated cells and at 12, 36, 48, and 60 h post-UV treatment. Bands 1 and 2, UV damage specific; P, free oligonucleotide. (B) Results of densitometer scanning of the damage-specific bands 1 and 2 plotted versus hours post-UV irradiation. Bands were scanned from the samples with an excess of unirradiated competitor. Dashed lines are minimum and maximum values for DDB protein band 1 in mock-treated cells. The mock-treated values for DDB protein band 2 were below the 0.1 peak area. Results are the means of values obtained from two DNA-binding assays with two different extract preparations.

probe (Fig. 1, oligonucleotide 3/4) in a DNA-binding assay analyzed by gel mobility shift. We typically observed one strong and up to five faint bands of binding activity; in addition, one strong and up to four faint bands were detected with the undamaged 3/4 probe (Fig. 2A, CV-1/0, no competitor). To inhibit nonspecific binding to the UV-damaged probe, we performed similar experiments in the presence of a 100-fold molar excess of unlabeled, undamaged oligonucleotide 3/4. The strong and weak bands which appear with undamaged DNA and some of the weaker bands which appear with UV-damaged DNA were inhibited, but the intense band (band 1) seen only with UV-damaged DNA (Fig. 2A, CV-1/0, –UV competitor) was not. This band, designated DDB activity, could be competed with a 100-fold molar excess of unlabeled, UV-irradiated DNA 3/4 (Fig. 2A, CV-1/0, +UV competitor), which suggested its binding specificity for UV-damaged DNA. Similar results were obtained with oligonucleotides of different sequences, indicating that modified DNA rather than a particular sequence is the target for the DDB activity (data not shown) (see below).

The biochemical nature of the DDB activity was determined by digesting CV-1 extracts with RNase A or proteinase K and then testing them in the binding assay. While RNase A digestion for 2 h at  $37^\circ\text{C}$  ( $50 \mu\text{g/ml}$ ) had no effect on DDB activity, 24 h of digestion of the extract with  $50 \mu\text{g}$  of proteinase K per ml at  $37^\circ\text{C}$  totally abolished binding. Cell extracts incubated at the same time without the addition of enzymes had no loss of DDB activity. Incubation of the extract for 1 h at  $65^\circ\text{C}$  fully inactivated DDB activity. The DDB activity thus appears to be a protein complex.

**DDB protein is induced in cells pretreated with agents which damage DNA or interfere with cellular DNA replication.** When *E. coli* cells are exposed to UV light, a large number of proteins are induced as a part of the SOS pathway, some of which are involved in DNA lesion recognition and repair. To test whether the DDB protein complex from mammalian

cells belongs to such a class of UV-inducible proteins, we irradiated CV-1 cells and prepared whole-cell extracts at different times after treatment. While the binding to undamaged oligonucleotide 3/4 increased only slightly over 60 h, the intensity of the damage-specific band 1 increased with time after UV irradiation, reaching a maximum at 48 to 60 h (Fig. 2A, lanes 3/4-UV, –UV, competitor). In extracts prepared at 36 to 60 h, a second damage-specific band (band 2) became readily visible.

Comparable results were obtained when nuclear extracts (18) instead of whole-cell extracts were tested for binding activity (Fig. 2B) (gels not shown). Nuclear extracts were prepared immediately after UV irradiation or at various times thereafter. In extracts prepared immediately after irradiation, constitutive DDB protein complex was undetectable (in the earlier experiments with whole-cell extracts, we had not assayed DDB protein immediately after UV treatment). The nuclear extracts displayed levels of protein binding to undamaged 3/4 DNA comparable to those of extracts prepared from mock-treated cells. Examination of the hypotonic supernatant for constitutive DDB protein complex, which could have leaked out from nuclei during homogenization, and reextraction of the nuclear pellet with 2 M NaCl did not reveal the activity. At 1 h after UV, however, the DDB protein became detectable, with about 10% of the mock-treated cells binding. Thereafter, the protein slowly accumulated in UV-treated cells through the next 2 to 3 days, reaching 200% above the levels in mock-treated cells. This accumulation (in both bands 1 and 2) could be prevented by incubating UV-treated cells immediately after irradiation with the protein synthesis inhibitors, dactinomycin (Fig. 3), or cycloheximide (not shown). The level of DDB protein also decreased somewhat in untreated cells after treatment with inhibitors. The levels of protein binding to undamaged probe 3/4 did not change after incubation with inhibitors (not shown). These results suggest that de novo

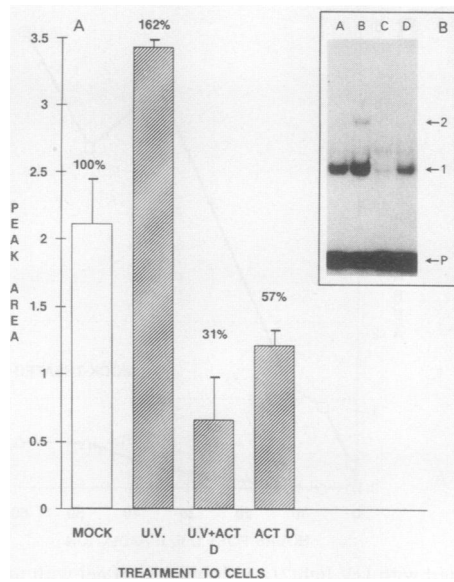


FIG. 3. Effect of dactinomycin treatment on the induction of the DDB protein complex in CV-1 cells. (A) Results of densitometer scanning of the damage-specific band 1 in extracts prepared from various treated cells. Percentages represent peak areas relative to control. Results are the means of values plus or minus standard deviations (error bars) obtained from two DNA-binding assays with two different extract preparations. (B) Mobility shift gel with UV-irradiated oligonucleotide 3/4 (8.6 J/m<sup>2</sup>) and nuclear binding proteins (6  $\mu$ g) prepared from either mock-treated cells (lane A) or cells treated with UV light (12 J/m<sup>2</sup>) (lane B), UV light plus dactinomycin (lane C), or dactinomycin alone (lane D). Binding assay was performed with a 100-fold molar excess of unirradiated competitor 3/4. Bands are as described in the legend to Fig. 2A.

synthesis of DDB protein occurs in UV-treated mammalian cells.

To determine whether the magnitude of enhanced binding is the result of the amount of UV damage to cellular DNA, we examined extracts prepared 48 h after UV irradiation of cells with 6, 12, 18, and 24 J/m<sup>2</sup>. There was a dose-dependent increase in the DDB activity of about 60, 120, 150, and 100%, respectively, above the levels in mock-treated cells (gel not shown). Although a dose-dependent difference in DDB activity of about twofold seems to be relatively small, it was reproducible. These results suggest that the amount of DNA damage might be the major determinant in the magnitude of DDB protein complex induction in UV-irradiated cells. Because of cytotoxicity at greater doses, the dose of 10 to 12 J/m<sup>2</sup>, which yields a colony-forming ability of about 10%, was used for further experiments.

To gain further insight into the nature of the inducing signal, we pretreated cells with (i) mitomycin C, a UV-mimetic compound which induces a mammalian SOS-like response (31) and triggers a pleiotropic effect known as the UV response (33); (ii) TPA, which induces a number of genes through responsive elements recognized by the TPA-regulated cellular transcription factor AP-1 (1); (iii) aphidicolin, a specific inhibitor of DNA polymerase alpha and delta; or (iv) low-serum growth medium, which arrests cell growth. Cell extracts were prepared 48 h after these treatments and tested for DDB protein. UV light, mitomycin C, and aphidicolin induced DDB protein by 90, 75, and 110%, respectively; TPA and 0.5% fetal calf serum medium had no effect. It thus appears that agents which directly damage

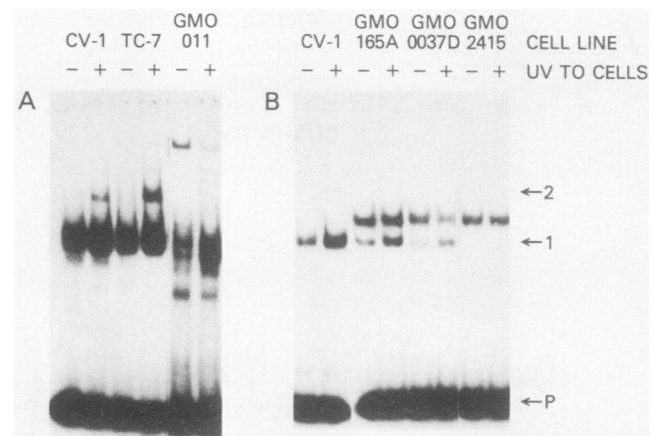


FIG. 4. Induction of DDB protein complex in UV-irradiated monkey kidney cells and human primary fibroblasts. Mobility shift gel with UV-irradiated oligonucleotide 3/4 (8.6 J/m<sup>2</sup>) and either whole-cell extracts (6  $\mu$ g) (A) or nuclear extracts (6,  $\mu$ g) (B) prepared from mock-treated (-) or UV-treated (12 J/m<sup>2</sup>) (+) cells. Extracts were prepared 48 to 72 h after cell treatment. Binding assays were carried out with a 100-fold molar excess of unirradiated competitor 3/4. Bands are as described in the legend to Fig. 2A.

cellular DNA (UV and mitomycin C) or directly inhibit cellular DNA replication (aphidicolin) are inducers of DDB protein complex.

Is a DDB protein complex present in cells other than monkey kidney? To answer this question, we tested a number of standard human cell lines, both primary and virus-transformed, for DDB activity. All repair-proficient cell lines tested and repair-deficient XP groups A, C, and D but not group E have detectable levels of constitutive DDB protein complex (Fig. 4) (data not shown). This finding is similar to the observation made by Chu and Chang (4) that XP group E cells lack binding activity specific for UV-damaged DNA. This activity was also lacking in UV-treated XP E cells (Fig. 4B), while three normal human cell lines had two- to sixfold more DDB activity 48 h after UV irradiation similar to monkey kidney cells (Fig. 4A and B). Taken together, these results suggest the general existence and inducibility of the DDB protein complex in cultured normal primate cells.

**What is the binding site of the DDB protein complex on UV-irradiated DNA?** UV irradiation of DNA at 254 nm and high fluences produces a variety of photoproducts, with pyrimidine dimers being the most frequent UV-induced DNA lesions (3, 20). The sequence of oligonucleotide 3/4 (Fig. 1) allows the formation of all possible photoproducts such as cyclobutane pyrimidine dimers, (6-4) dimers, purine dimers, cytosine and thymine glycol, and interstrand cross-links. In addition, the presence of UV-induced photoproducts is known to affect the conformation of the DNA by inducing bending, distortion, and possibly single-stranded short patches opposite the bulky lesions. To determine which lesion(s) might be the target(s) for the DDB protein complex, we prepared different binding substrates and used them as probes or competitors. Table 1 summarizes the results. The DDB protein complex is not detected when the probe is undamaged single-stranded DNA or double-stranded DNA, UV-irradiated single-stranded DNA, hydrogen peroxide- or osmium tetroxide-treated double-stranded DNA, or double- or triple-base-pair mismatched DNA. In addition, all of these substrates are poor competitors in the

TABLE 1. Activities of different DNAs as binding or competing substrates for DDB protein complex (band 1)

DNA	Treatment	Binding <sup>a</sup> to DNA	Competition for binding to 3/4-UV <sup>b</sup>
<b>Double-stranded oligonucleotides</b>			
3/4	None	No	No (1,000)
	UV	Yes	Yes (10)
	UV + 60°C	Yes	NT
23/24	H <sub>2</sub> O <sub>2</sub> , OsO <sub>4</sub>	No	No (100)
	None	No	No (500)
23/4	None	No	No (500)
	UV	Yes	Yes (500)
<b>Mismatched oligonucleotides</b>			
3/134	None	No	No (250)
	UV	± Yes	Yes (250)
23/4	None	No	No (125)
	UV	± Yes	Yes (125)
<b>Single-stranded oligonucleotides</b>			
3	None	No	No (200)
	UV	No	No (200)
<b>Polydeoxynucleotides</b>			
poly(dA) · poly(dT)	None	NT	No (1,000)
	UV	NT	± Yes (1,000)
poly(dG) · poly(dC)	None	NT	No (1,000)
	UV	NT	± Yes (1,000)
poly(dI-dC)/poly(dI-dC)	UV	NT	No (1,000)

<sup>a</sup> Binding activity: Yes, one or two UV-specific bands are retarded; ± Yes, weak specific binding; No, retarded bands not UV-specific; NT, not tested.  
<sup>b</sup> Competing activity: molar ratio of binding versus competing DNA. Values in parentheses represent the molar ratio with minimum (Yes) or maximum (No) concentrations tested; ± Yes, partial competition. Results are from one to three experiments for each DNA.

DDB protein-UV-irradiated double-stranded DNA interaction. UV-irradiated synthetic polynucleotides with adjacent pyrimidines, poly(dA) · poly(dT) and poly(dG) · poly(dC), partially compete for binding but only at a high molar ratio (1,000-fold).

We asked whether the presence of UV-induced interstrand cross-links is necessary for the detection of the DDB protein complex. To avoid cross-links, we prepared the probes by separately irradiating oligonucleotides 3 and 4 (upper and lower strands of probe 3/4, respectively) (Fig. 1) and annealing them to unirradiated complementary strands 4 and 3, respectively. These oligonucleotides were as efficient as UV-irradiated double-stranded oligonucleotide 3/4 as substrates for the binding protein if the UV-irradiated strand was strand 3 (pyrimidine-rich strand) but not strand 4 (purine-rich strand). These results suggest that the UV-induced interstrand cross-links are not a major target for the DDB protein complex and, furthermore, that UV photoproducts of pyrimidines rather than purines are necessary for binding.

The role of adjacent pyrimidines became particularly apparent when we examined the binding of UV-irradiated oligonucleotide 190/200 (Fig. 1). This sequence is similar in size and in base content to that of oligonucleotide 3/4 but has no adjacent pyrimidines. We detected a 10-fold weaker DDB activity than that detected with UV-irradiated oligonucleotide 3/4 (Fig. 5A). Oligonucleotide 190/200, however, could not inhibit the DDB protein-3/4-UV DNA interaction at a 500-fold molar excess and might represent another DDB

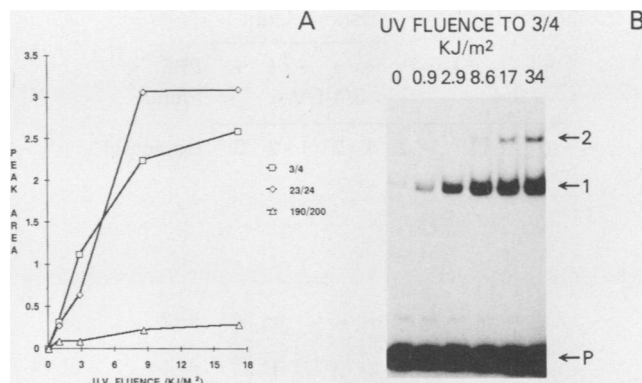


FIG. 5. Effect of UV fluence to the probes on the DDB activity. (A) Results of densitometer scanning of retarded band 1 plotted versus increasing UV fluences to three different oligonucleotides (sequence data in Fig. 1). Assays were performed with 4.6 µg of cell extract proteins prepared from UV-treated cells. (B) Mobility shift gel with oligonucleotide 3/4 and whole-cell extracts (4.6 µg) prepared from UV-treated cells. Note the dose-dependent appearance of bands 1 and 2. P, Free oligonucleotide.

protein complex which cannot be distinguished by mobility shift from band 1. The oligonucleotides with adjacent pyrimidines but a sequence entirely different from that of oligonucleotide 3/4 were effective both as probes and as competitors for DDB protein complex (data not shown).

To examine whether the DDB protein complex is UV dose dependent, oligonucleotide 3/4 was irradiated with increasing UV fluences and used in the binding assay. Damage-specific band 1 becomes visible at a fluence of 0.9 kJ/m<sup>2</sup>, which yields an average of 0.8 cyclobutane pyrimidine dimers per double-stranded DNA (calculated as described by Protić-Sabljić and Kraemer [27]) (Fig. 5B). The intensity of band 1 increases up to 10-fold with the UV dose of 8.6 kJ/m<sup>2</sup> and shows a linear increase in binding with an increase in UV dose (Fig. 5A, probe 3/4). The damage-specific band 2 becomes visible at UV fluences of about 8 kJ/m<sup>2</sup>. Oligonucleotide 3/4 with a triple-base substitution at the -TTT- site, 23/24 (Fig. 1), shows similar dose-dependent binding as 3/4. When these probes were analyzed on denaturing polyacrylamide gels, we detected a dose-dependent increase in the number of T4 polymerase-exonuclease-inhibiting sites (6), hot alkali labile sites (20), and interstrand cross-links (data not shown). T4 polymerase-exonuclease [an enzyme which is inhibited by the presence of both cyclobutane and (6-4) pyrimidine dimers in DNA] digestion generated bands whose intensities and positions did not change significantly between 2.9 and 8.6 kJ/m<sup>2</sup>. We observed, however, a dose-dependent increase in the intensity of fragments after treatment of UV-irradiated (0.9 to 34 kJ/m<sup>2</sup>) oligonucleotide 3/4 with hot alkali. This may indicate a linear induction of (6-4) dimers with fluences up to 34 kJ/m<sup>2</sup> and saturation in the induction of the cyclobutane type of pyrimidine dimer with a UV fluence of about 5 kJ/m<sup>2</sup>, as previously observed for other DNA fragments (3). A positive correlation between the intensity of the DDB protein complex and the induction of hot alkali labile sites suggested that the (6-4) dimer rather than the cyclobutane type of pyrimidine dimer may be the major target site.

To distinguish between the two types of dimers, we tested whether specific monomerization of cyclobutane pyrimidine dimers by photoreactivation will affect the binding of UV-irradiated DNA. With a probe prepared in the absence of

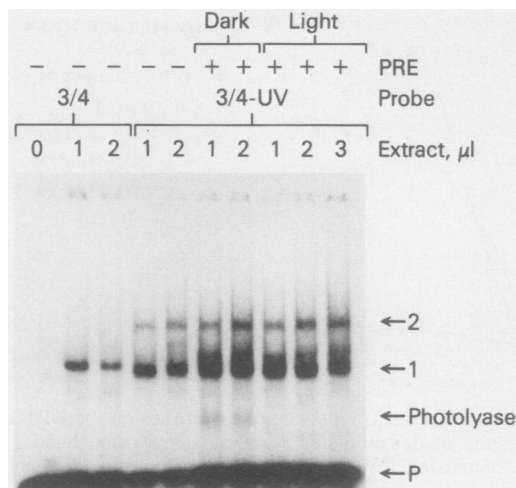


FIG. 6. Effect of photoreactivation (PRE) on the binding of the DDB protein complex to UV-irradiated DNA. Oligonucleotide 3/4 was irradiated with 8.6 J of UV light per  $m^2$  (3/4-UV) and incubated with *E. coli* photolyase in the presence (+) or absence (-) of photoreactivating light. These probes were then tested as binding substrates for DDB protein complex (bands 1 and 2) with whole-cell extracts (6.6  $\mu g/\mu l$ ) from UV-treated CV-1 cells. P, Free oligonucleotide.

photoreactivating light, in addition to the UV-specific bands 1 and 2 from cell extracts, a new band representing a complex of *E. coli* photolyase and UV-irradiated probe is visible (Fig. 6). This band disappears after photoreactivation, indicating the loss of photolyase binding sites (cyclobutane pyrimidine dimers [30]). However, the DDB protein was not affected. If the DDB protein binds with lower affinity to the cyclobutane type of pyrimidine dimer than to (6-4) dimers, we could not detect the change in the intensity of the DDB protein using the above approach. Therefore, we prepared binding substrates with 1 or 4 -TT- cyclobutane dimers (Fig. 1, 11<sup>^</sup>/230 and 11<sup>^</sup>/44). Using either probe, we could not detect DDB protein complex with CV-1 whole-cell extracts or partially purified protein fractions (Abramic et al., unpublished data), although the probes could bind *E. coli* photolyase. If the -TT- dimer probes were UV irradiated, the DDB protein complex was detected with a dose dependency similar to that of the same UV-irradiated probes without -TT- dimers (gels not shown). These findings suggest that the cyclobutane type of -TT- pyrimidine dimer may not be a high-affinity target for this DDB protein complex.

## DISCUSSION

We have identified a DDB protein complex in primate cells which has a high affinity for UV-damaged DNA. Chu and Chang (4) recently reported that normal human cells and all repair-deficient XP groups except group E contain constitutive nuclear factors that bind to UV-damaged DNA. A careful examination of their results and a comparison with our data revealed that one of the nuclear factors which they named DDBF2 might be analogous to our damage-specific band 1: it shows high affinity for UV-damaged DNA and is lacking in XP group E cells. A second band, designated DDBF1 by Chu and Chang, with lower affinity for UV-damaged DNA and which is apparently also missing in XP E cells is similar to the band which we see in extracts from human cells between damage-specific bands 1 and 2 (Fig. 4). In contrast to their observation, we found this band to be

present also in XP group E cells (Fig. 4B). Since we used the same XP cell line (GM02415) as Chu and Chang did to prepare nuclear extracts, it is possible that the amount of nonspecific competitor DNA they used in the binding assay masked this DNA-binding activity. The damage-specific band 2, which we detected in extracts from both monkey and human cell lines, was apparently not detected by Chu and Chang. This band appears if the following conditions for the binding reaction are modified: a greater quantity of constitutive proteins is used, the same quantity of induced proteins is used, and a DNA probe is irradiated with higher doses of UV light. Competition analysis indicated that band 2 has an affinity for UV-irradiated DNA similar to that of band 1 which, taken together, would suggest that band 2 represents a multimer of band 1. Purification of these binding activities should clarify the origins of these bands.

What is the binding substrate for this DDB protein complex? Our characterization of DNA-binding substrates suggests that the most likely UV lesion responsible for DDB protein complex binding is the (6-4) pyrimidine dimer. However, Chu and Chang proposed, on the basis of UV dose-dependence data, that the UV-damage-specific DNA-binding activity which they detected in human cell extracts in a similar type of assay is specific for cyclobutane pyrimidine dimers rather than for other types of UV photoproducts (4). Our results, nonetheless, suggest that the -TT-cyclobutane pyrimidine dimer is not a high-affinity target for this DDB protein complex. It is possible, although not probable, that a different distribution or type of cyclobutane pyrimidine dimer substrate alters the binding affinity for this protein complex.

This study has shown that the DDB protein complex is apparently synthesized de novo in cultured primate cells after treatment with UV light, with an increase in DNA binding of two- to sixfold. Enhanced DDB activity has also been observed with cells pretreated with mitomycin C and aphidicolin but not TPA. These findings correlate with our earlier observation that repair of UV-irradiated expression vectors is enhanced in CV-1 cells after UV and mitomycin C treatment but not after TPA treatment (26) (M. Protić, unpublished data). It appears that enhanced repair of UV-damaged plasmid DNA and enhanced DDB protein synthesis might be elicited by the same induction pathway in pretreated cells. What is the inducing signal for the DDB protein complex? Agents which directly damage cellular DNA (UV and mitomycin C) or directly inhibit cellular DNA replication (aphidicolin) are inducers of DDB protein. The arrest of cell growth per se might not be sufficient for the induction because both TPA (which transiently arrests cells in G2 phase) and serum starvation (which arrests cells in G0 phase) had no effect on this DDB protein complex. There is a possibility, however, that the phase of the cell cycle in which cells are arrested affects synthesis of this protein complex, since both types of inducing agents arrest cells in S phase. Further studies are needed to understand the role of the cell cycle in the regulation of this binding activity. Because the quantity of residual binding activity immediately after UV irradiation (M. Protić, unpublished data) and the magnitude of enhanced DDB protein depend on the dose of UV to the cells, we believe that the extent of cellular DNA damage or persistence of DNA replication blocks is likely to be a major factor in determining the inducing signal.

The DDB protein complex could not be detected in either mock-treated or UV-pretreated XP group E cells (this study). XP is a human genetic cancer-prone disease. XP group E cells are hypersensitive to killing by UV light and



have a reduced rate of (6-4) photoproduct repair in total cellular DNA (for a review, see reference 23). This repair deficiency might be the consequence of the group E defect in the DNA-binding activity specific for (6-4) dimers. This would imply that the DDB protein complex we have identified in primate cells might have a role in DNA repair, possibly at the stage of lesion recognition and formation of a repair complex.

The DDB protein complex described here is the first such inducible, damage-specific protein complex identified in primate cells. According to its DNA-binding characteristics and substrate specificity, the DDB protein complex we identified appears to be different from the DDB proteins previously isolated from HeLa cells (16, 34) or human placenta (10, 25) and from other types of DNA-binding proteins such as histones and transcription factors. This protein complex also appears to belong to a different class from those described by Glazer et al. (14), who found UV-inducible DNA-binding proteins in HeLa cells in the presence of either dactinomycin or cycloheximide and which have no specificity for UV-damaged DNA. Similarly, the DDB protein complex is unlikely to be one of the induced cellular proteins detected in human fibroblasts by Schorpp et al. (33), since these proteins are inducible by both UV and TPA and are apparently cytoplasmic. The enhanced DDB activity in UV-irradiated CV-1 cells might be associated with a greater removal of (6-4) dimers and thus be responsible for the enhanced repair of UV-irradiated expression vectors which we previously observed in these cells (26). It is interesting that the repair of (6-4) photoproducts correlates with split-dose recovery in UV-irradiated normal rodent cells (24). Future studies, e.g., in vitro repair synthesis using purified DDB protein(s) and defined DNA substrates, are needed to test this hypothesis.

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